ARTICLE

Adenovirus-Mediated p53 Gene Transfer in Advanced Non-Small-Cell Lung Cancer

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Background: **Preclinical studies in animal models have demonstrated tumor regression following intratumoral administrati n of an adenovirus vector containing wild-type p53 complementary DNA (Ad-p53). Therefore, in a phase I clinical trial, we administered Ad-p53 to 28 patients with non-small-cell lung cancer (NSCLC) whose cancers had progressed on conventional treatments.** *Methods:* **Patients received up to six, monthly intratumoral injections of Ad-p53 by use of computed tomography-guided percutaneous fine-needle injection (23 patients) or bronchoscopy (five patients). The doses ranged from 10^6 plaque-forming units (PFU) to 10^{11} PFU.** *Results:* **Polymerase chain reaction (PCR) analysis showed the presence of adenovirus vector DNA in 18 (86%) of 21 patients with evaluable posttreatment biopsy specimens; vector-specific p53 messenger RNA was detected by means of reverse transcription–PCR analysis in 12 (46%) of 26 patients. Apoptosis (programmed cell death) was demonstrated by increased terminal deoxynucle tide transferase-mediated biotin uridine triphosphate nick-end labeling (TUNEL) staining in posttreatment biopsy specimens from 11 patients. Vector-related toxicity was minimal (National Cancer Institute's Common Toxicity Criteria: grade 3 = one patient; grade 4 = no patients) in 84 courses of treatment, despite repeated injections (up to six) in 23 patients. Therapeutic activity in 25 evaluable patients included partial responses in two patients (8%) and disease stabilization (range, 2–14 months) in 16 patients (64%); the remaining seven patients (28%) exhibited disease progression.** *Conclusions:* **Repeated intratumoral injections of Ad-p53 appear to be well tolerated, result in transgene expression of wild-type p53, and seem to mediate antitumor activity in a subset of patients with advanced NSCLC. [J Natl Cancer Inst 1999;91:763–71]**

The p53 gene (also known as TP53) encodes a 593-amino acid ph sphoprotein that plays a critical role in cell cycle regulation and control of apoptosis *(1–3)*. p53 gene mutations have been associated with tumor progression and the development of chemotherapy and radiation therapy resistance *(4–6)*. The development of gene transfer technology has allowed the transduction of cancer cells with wild-type p53 (wt-p53). Intratumoral injection of retroviral or ad noviral wt-p53 constructs in animal models results in tumor regression in a variety of different tumor histologies, including non-small-cell lung cancer (NSCLC), leukemia, glioblastoma, and breast, liver, ovarian, colon, and kidney cancers *(7–13)*. Furthermore, Roth et al. *(14)* demonstrated the safety and feasibility of using a retroviral wt-p53 construct in patients with advanced NSCLC. In that trial, tumors regressed in three of seven evaluable patients after bronchoscopic or computed tomography (CT)-guided injection of retroviral wt-p53.

The clinical use of retrovirus vectors is limited, however, by difficulties in transducing nonreplicating cells and producing high titers of virus. Adenoviruses, on the other hand, are large, double-stranded DNA viruses with a tropism for lung cancer cells *(15)*. Furthermore, they are capable of transducing nonreplicating cells and can be grown to high titers *in vitro*, which allows for their potential clinical utility *(16)*. We, therefore, designed a phase I clinical trial using an adenovirus vector containing wt-p53 complementary DNA (cDNA) to treat patients with advanced NSCLC whose cancers had progressed on conventional treatments.

Patients and Methods

Protocol approval. The protocol used was approved by the Biosafety and Surveillance Committees/Institutional Review Board of the participating institutions, the Recombinant DNA Advisory Committee of the National Institutes of Health, and the U.S. Food and Drug Administration *(17)*.

Gene transfer vector. The construction and generation of Ad-p53 were reported previously *(18)*. Briefly, E1-deleted replication-defective recombinant

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See "Notes" following "References."

adenovirus was constructed with the use of a modified type 5 adenovirus genome. The cytomegalovirus (CMV) promoter was used to drive transcription of human wt-p53 cDNA. Ad-p53 was supplied by Introgen Therapeutics, Inc.

Eligibility criteria and treatment protocol. Patients with histologically proven NSCLC were enrolled in the trial. All patients had unresectable tumors and either were unable to receive primary external beam radiation therapy or had a recurrence after such therapy. Patients were also eligible if they did not respond to or relapsed after chemotherapy. Patients had either an endobronchial tumor that was accessible by the bronchoscope with some clinical evidence of bronchial obstruction, advanced local–regional cancer that was unresectable, or isolated metastases whose regression or stabilization would offer potential benefit to the patient. Written informed consent was obtained from all patients stating that they were aware of the investigational nature of this study, in keeping with institutional policies. Pretreatment tumor biopsies demonstrating overexpression of the p53 protein by the criteria of Nishio et al. *(19)* were required for entry in the protocol. Mutations in the p53 gene were identified by single-strand conformation polymorphism (SSCP) analysis and DNA sequencing of a tumor biopsy specimen as described previously *(20)*. All mutations were reconfirmed by repeated sequencing or SSCP analysis of a second independent polymerase chain reaction (PCR) reaction. Patients were not treated on protocol until 4 weeks after completing systemic or local therapy. The preclinical safety studies and treatment protocol have been described previously *(21,22)*. Ad-p53 was diluted in phosphate-buffered saline and administered by needle injection directly into the tumor, either percutaneously or bronchoscopically. For lesions at least 4 cm in the largest diameter, the final volume given was 10 mL; for lesions with a diameter of less than 4 cm, the final volume given was 3 mL. The entire volume was injected at a single site. Patients were treated monthly for up to six injections of Ad-p53. Doses were escalated from 10^6 plaque-forming units (PFU) to 10^{11} PFU in one-half or one log increments (Table 1).

Toxicity and response. The toxic effects of therapy were evaluated according to the National Cancer Institute's Common Toxicity Criteria *(23)*. Response to therapy was assessed by chest roentgenogram or CT scans before each course of treatment, by use of standard criteria *(14)*. Responses were confirmed by two evaluations taken 4 weeks apart. Patients were evaluable for response if they had received at least one course of therapy followed by an appropriate radiograph to document response. Response criteria were defined as follows: 1) complete response, i.e., disappearance of all clinical evidence of tumor by physical examination, roentgenography, and CT (or magnetic resonance imaging) scans for a minimum of 4 weeks; 2) partial response, i.e., a 50% or greater decrease in the sum of the products of the perpendicular diameters of measurable lesions for a minimum of 4 weeks and no simultaneous increase of at least 25% in the size of any lesion or the appearance of any new lesion; 3) stable disease, i.e., any variation of the indicator lesion not meeting the criteria of a complete or partial response or progression; and 4) progressive disease, i.e., an increase of at least

Table 1. Characteristics of patients with non-small-cell lung cancer (NSCLC), characteristics of their tumors, prior therapy, and response to treatment with intratumoral injection of Ad-p53, an adenovirus vector carrying wild-type p53 complementary DNA

Patient*	Age, y	Sex	Histology†	Prior surgery	Prior chemotherapy courses‡	Prior radiation therapy§	Injection site‖	Baseline measurement, cm	Method of injection¶	Viral dose, plaque-forming units	No. of courses	Response#*
A	45	Female	Squamous	No	5	6300	Lung	4 × 6	CT	10^6	4	Stable
B	61	Male	Squamous	Yes	2	12 420	Lung	10 × 9	CT	10^6	2	Stable
C	75	Female	Adeno	No	4	None	Bronchus	5 × 6	Bronch	10^6	1	Progression
D	58	Male	Squamous	No	9	6000	Subcarinal nodes	4 × 2	CT	10^7	3	Progression
E	43	Female	Adeno	Yes	3	7500	Liver	2 × 2	CT	10^7	2	Progression
F	58	Female	Large cell	Yes	0	6000	Axilla	3 × 6	CT	10^7	1	Not evaluable**
G	42	Male	Adeno	No	0	None	Liver	4.5 × 6	CT	10^8	3	Stable
H	61	Male	Adeno	No	3	6120	Liver	5 × 6	CT	10^8	4	Stable
I	65	Female	Sarco	No	3	None	Chest wall	6.5 × 5	CT	10^8	2	Stable
J	71	Female	Squamous	Yes	0	6480	Lung	5 × 5	CT	10^9	2	Progression
K	72	Female	Large cell	No	6	6600	Lung	4 × 5	CT	10^9	6	Partial response
L	78	Male	Adeno	Yes	2	6000	Liver	4 × 5	CT	10^9	3	Stable
M	68	Female	Squamous	No	2	Yes	Lung	3.5 × 5	CT	3×10^9	4	Stable
N	72	Female	Adeno	Yes	0	8000	Lung	6.5 × 7	CT	3×10^9	2	Stable
O	70	Female	Adeno	Yes	2	6000	Bronchus	ND††	Bronch	3×10^9	6	Partial response
P	52	Male	Adeno	No	6	6000	Bronchus	3.9 × 6.3	Bronch	3×10^9	2	Stable
Q	65	Female	Adeno	Yes	0	11 000	Lung	3.5 × 3.5	CT	10^{10}	5	Stable
R	68	Female	Large cell	No	0	None	Lung	5 × 5	CT	10^{10}	2	Stable
S	55	Male	Squamous	No	3	6000	Lung	5 × 5	CT	10^{10}	5	Stable
T	46	Female	Adeno	Yes	4	4000	Lung	5.3 × 5.3	CT	3×10^{10}	2	Progression
U	71	Male	Squamous	Yes	2	13 914	Bronchus	ND††	Bronch	3×10^{10}	1	Not evaluable**
V	67	Female	Adeno	No	3	None	Lung	4 × 2.5	CT	3×10^{10}	6	Stable
W	55	Female	Adeno	No	4	None	Lung	3.8 × 4.5	CT	10^{11}	3	Progression
X	75	Male	Squamous	No	5	6300	Lung	6.5 × 3	CT	10^{11}	6	Stable
Y	61	Male	Squamous	No	2	6800	Bronchus	ND††	Bronch	10^{11}	1	Not evaluable**
Z	74	Male	Large cell	No	0	8000	Liver	9.3 × 8.3	CT	10^{11}	2	Stable
AA	62	Female	Squamous	No	2	6300	Lung	5 × 6.5	CT	10^{11}	3	Stable
BB	67	Female	Adeno	No	5	None	Chest wall	2 × 4	CT	10^{11}	1	Progression

*Sequential letters were assigned during manuscript editing to ensure confidentiality of patients; the letters do not represent identifiers used during the trial.

†All tumors were histologically confirmed before treatment as viable NSCLC; squamous = squamous cell carcinoma; adeno = adenocarcinoma; large cell = large-cell carcinoma; and sarco = sarcomatoid subset of NSCLC.

‡Number of chemotherapy courses given at least 3 months prior to Ad-p53 treatment.

§Centigray (cGy) of external-beam radiation therapy given at least 3 months prior to Ad-p53 therapy.

‖Location of indicator lesion injected with Ad-p53.

¶Mode of delivery of intratumoral Ad-p53 during monthly treatments; CT = computed tomography (CT)-guided injection; Bronch = bronchoscopic injection.

#Complete response = complete disappearance of tumor as judged by CT scan and physical examination for a minimum of 4 weeks; partial response = decrease ≥50% in size of tumor for minimum of 4 weeks; progression = increase ≥25% in size of tumor; stable = any variation in size not meeting criteria of complete response, partial response, or progression.

**Not evaluable because patients died of non-treatment-related causes prior to 30-day follow-up CT scan.

††ND = not determined because it was an endobronchial tumor.

25% in the size of a bidimensionally or unidimensionally measurable lesion, a clinically significant increase in the size of noninjected disease, or the appearance of any unequivocal new lesion.

Terminal deoxynucleotide transferase (TdT)-mediated biotin uridine triphosphate nick-end labeling (TUNEL) assay for DNA fragmentation. Pretreatment (immediately before) and posttreatment (3 days after) tumor biopsy specimens were obtained by core biopsies of the vector-injected tumor after each course of treatment (Fig. 1). The TUNEL assay was a modification of a previously described technique *(24,25)*. Slides were counterstained with 0.4% methylene green. Negative controls were performed by omitting TdT from the buffer solution, and positive controls included analysis of deoxyribonuclease-treated slides. Corresponding hematoxylin–eosin slides were evaluated for the presence of an inflammatory cell infiltrate and were graded on a scale of 1–4. All histology slides were coded and read blinded by a single observer, who had no knowledge of the patients, biopsy sequence, or the clinical status.

Reverse transcription (RT)–PCR and DNA PCR. Total RNA extraction, RT, PCR amplification, and blot hybridization were performed by a modification of a previously described technique *(26,27)*. A nested PCR procedure was used, with vector-specific primers CMV3 (5′-GGTGCATTGGAACGCGGATT-3′) and Rev Ex3 (5′-CAAATCATCC ATTGCTTGGGA-3′) used for the first round and CMV3 and RN3 (5′-GGGGACAGAACGTTGTTTTC-3′) used for the second round of amplification. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (GAPDH-S [5′-CAGCCGAGCCACATC-3′] and GAPDH-AS [5′-TGAGGCTGTTGTCATACTTCT-3′]) were used as a positive reaction control, and saline was used as a negative control. All samples underwent PCR amplification without prior reverse transcriptase treatment to test for completeness of deoxyribonuclease digestion. RT–PCR amplification was not performed if DNA PCR was negative for adenoviral DNA or biopsy samples were inadequate for RNA evaluation. DNA PCR was performed on DNA extracted from biopsy specimens by use of the primers described above.

Statistical methods. Because of the small sample size, descriptive statistics were reported in tabular form. Ninety-five percent confidence intervals (CIs) were constructed to estimate the pretreatment apoptotic index (AI). Patients were considered to have increased apoptotic activity if the posttreatment AI was greater than the upper end of the 95% CI of the pretreatment AI. The overall survival was calculated by the Kaplan–Meier estimate. Survival time was defined as time from study entry to death or date of last follow-up.

RESULTS

Patient and Tumor Characteristics

Twenty-eight NSCLC patients (17 females and 11 males) with a median age of 65 years (range, 42–78 years) were entered in the study (Table 1) beginning October 24, 1995, until December 8, 1997. Partial information on 12 of these patients was reported previously *(28)*, together with information on nine patients who received Ad-p53 and cisplatin in a companion study. A total of 84 courses were administered, and the date of last follow-up was March 31, 1998 (median follow-up of 421 days). Patients had documented p53 mutations and histologically determined, viable non-small-cell lung carcinoma as judged by pretreatment tumor biopsies. Patients were treated either percu-

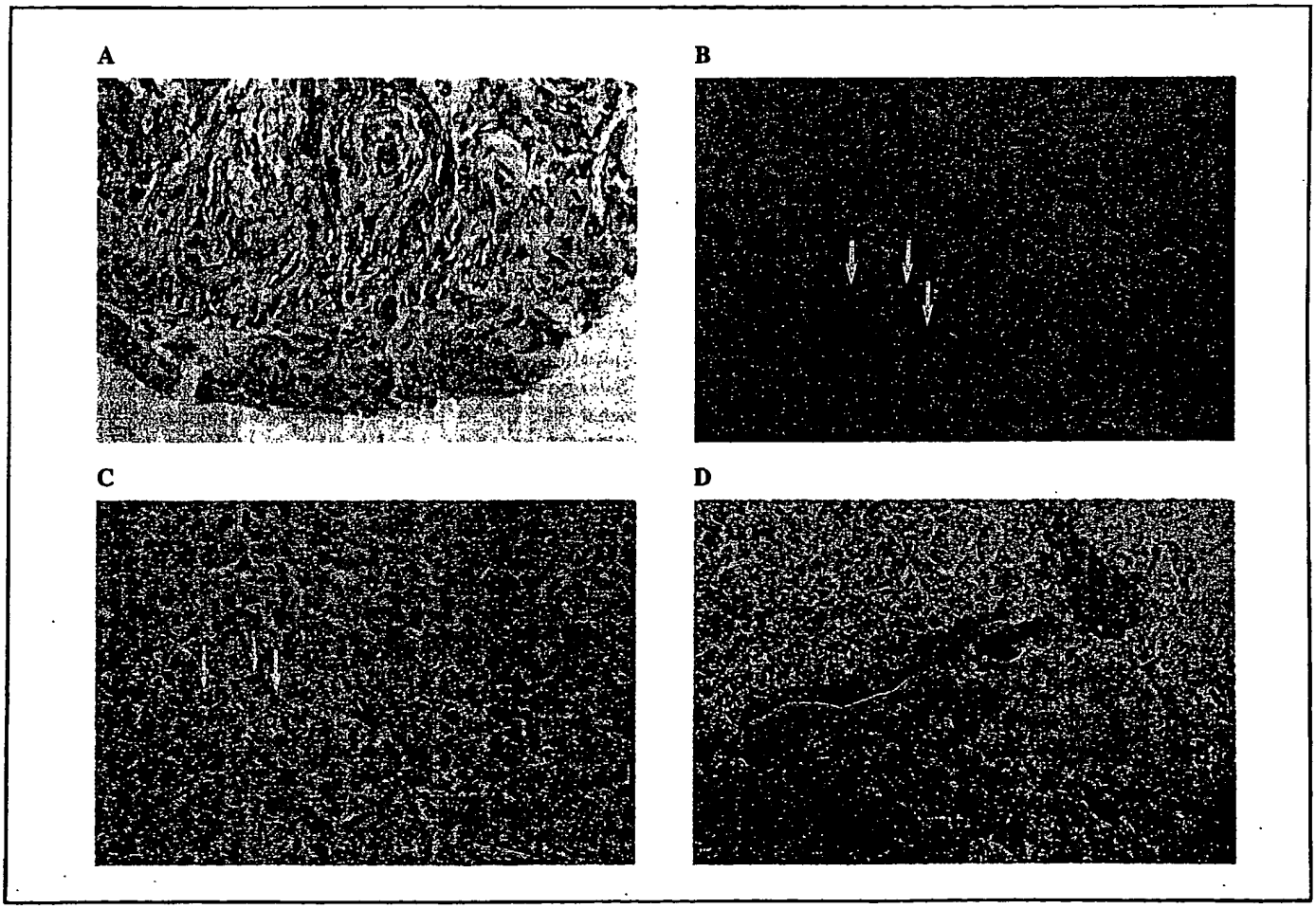

Fig. 1. Staining by terminal deoxynucleotide transferase (TdT)-mediated biotin uridine triphosphate nick-end labeling (TUNEL) technique of left upper lobe tumor biopsy specimens from patient O. Pretreatment (A) and 3 days after injection of 3×10^9 plaque-forming units of Ad-p53 (B). Positive control reactions (C) were performed with deoxyribonuclease-treated sections, while negative control reactions (D) omitted TdT from buffer solution. Arrows outline region of positive nuclear staining, which reflects DNA fragmentation consistent with cells undergoing apoptosis (original magnification ×400).

taneously with the use of CT guidance (23 patients) or bronchoscopically (five patients). Before entry in the study, all patients demonstrated progressing primary or metastatic lesions that had failed to respond to conventional therapies, including surgery in 10 patients (36%), radiation therapy in 25 (89%), and chemotherapy in 21 (75%). Vector-injected tumors were located in the lung parenchyma in 14 patients (50%), chest wall in two patients (7%), bronchus in five patients (18%), liver in five patients (18%), axilla in one patient (4%), and subcarinal node in one patient (4%).

Assessment of Gene Transfer

We performed PCR analysis with vector-specific primers for the adenovirus and p53 sequences to differentiate between vector-transduced p53 and cellular p53 (Table 2). No pretreatment tumor biopsy samples showed evidence of adenoviral p53 DNA (DNA–PCR) or messenger RNA (mRNA) (RT–PCR) sequences. DNA extracted from 18 of 21 evaluable tumors showed vector-specific adenovirus sequences. All patients who received more than 10^6 PFU showed evidence of adenovirus sequences by DNA–PCR in their posttreatment specimens. Vector-specific mRNA p53 sequences were detected by RT–PCR in 12 of 26 evaluable specimens. Transgene expression of p53 was noted in nine of 16 patients treated at doses above 10^9 PFU, as opposed to only three of 10 patients treated with 10^9 PFU or less. Transgene expression of p53 occurred after initial and subsequent treatments at all dose levels above 10^6 PFU.

The mean pretreatment AI was 3.6% (95% CI = 1.5%–5.8%). After administration of Ad-p53, 11 of 24 evaluable patients fell outside the pretreatment 95% CIs with apoptotic indices of 7%–87% in posttreatment tumor biopsy samples (Table 2). No consistent change in inflammatory cell infiltration was seen after Ad-p53 treatment in posttreatment tumor biopsy samples (data not shown).

Adverse Events

Vector-related adverse events were minimal (Table 3). No grade 4 toxicity was seen and grade 3 vector-related toxicity was limited to one incident of nausea after Ad-p53 injection. CT-guided administration of vector resulted in six pneumothoraces that were treated with percutaneous placement of a pigtail catheter in two patients and observation in four patients. Injection site pain was noted during 13 courses (15.5%) and was resolved with oral pain medications in all patients. Four incidents of

Table 2. Assessment of gene transfer and expression in biopsy samples of non-small-cell lung cancer following treatment with Ad-p53, an adenovirus vector carrying wild-type p53 complementary DNA

Patient*	Viral dose, plaque-forming units	No. of courses	DNA–PCR†	RT–PCR†	TUNEL, % positive cells‡	Response§
A	10^6	4	–	–	4	Stable
B	10^6	2	–	–	7‖	Stable
C	10^6	1	–	–	NT¶	Progression
D	10^7	3	+ (2)	NT¶	NT¶	Progression
E	10^7	2	+ (2)	+ (2)	2	Progression
F	10^7	1	+ (1)	+ (1)	7‖	Not evaluable#
G	10^8	3	+ (3)	+ (3)	8‖	Stable
H	10^8	4	+ (1, 2)	–	50‖	Stable
I	10^8	2	+ (1, 2)	–	10‖	Stable
J	10^9	2	+ (1, 2)	–	NT¶	Progression
K	10^9	6	+ (1, 3, 5, 6)	–	4	Partial response
L	10^9	3	+ (3)	–	4	Stable
M	3×10^9	4	+ (3, 4)	+ (4)	12‖	Stable
N	3×10^9	2	+ (1, 2)	+ (1)	4	Stable
O	3×10^9	6	NT¶	+ (1, 2, 6)	46‖	Partial response
P	3×10^9	2	NT¶	–	3	Stable
Q	10^{10}	5	+ (2, 4, 5)	–	50‖	Stable
R	10^{10}	2	+ (1, 2)	+ (1, 2)	NT¶	Stable
S	10^{10}	5	+ (1, 2, 3, 4)	–	7‖	Stable
T	3×10^{10}	2	NT¶	+ (1)	4	Progression
U	3×10^{10}	1	NT¶	–	0	Not evaluable#
V	3×10^{10}	6	+ (1, 2, 3)	–	87‖	Stable
W	10^{11}	3	NT¶	+ (2, 3)	7‖	Progression
X	10^{11}	6	+ (1, 3, 4, 5, 6)	–	0	Stable
Y	10^{11}	1	NT¶	+ (1)	2	Not evaluable#
Z	10^{11}	2	NT¶	–	3	Stable
AA	10^{11}	3	+ (2, 3)	+ (2)	1	Stable
BB	10^{11}	1	+ (1)	+ (1)	1	Progression

*Sequential letters were assigned during manuscript editing to ensure confidentiality of patients; the letters do not represent identifiers used during the trial.

†Course number during which DNA–polymerase chain reaction (PCR) or reverse transcription (RT)–PCR was positive is given in parentheses.

‡Maximum percentage of cells staining positive by terminal deoxynucleotide transferase-mediated biotin uridine triphosphate nick-end labeling (TUNEL) posttreatment. Mean pretreatment apoptotic index was 3.6% with 95% confidence interval = 1.5%–5.8%.

§Partial response = decrease ≥50% in size of tumor for minimum of 4 weeks; complete response = complete disappearance of tumor as judged by computed tomography scan and physical examination for a minimum of 4 weeks; progression = increase ≥25% in size of tumor; stable = any variation in size not meeting criteria of complete response, partial response, or progression.

‖Posttreatment apoptotic index above 95% confidence interval of pretreatment apoptotic index.

¶NT = not tested because of insufficient quantity or quality of biopsy specimen.

#Not evaluable because patients died of non-treatment-related causes prior to 30-day follow-up computed tomography scan.

Table 3. Adverse events associated with Ad-p53 (adenovirus vector) gene therapy in patients with non-small-cell lung cancer*

Adverse event	No. of courses†	Grade 1‡	Grade 2‡	Grade 3‡	Grade 4‡	Total§
Fever	84	13 (15.5)	10 (11.9)	0	0	23 (27.4)
Injection site pain	84	6 (7.1)	6 (7.1)	1 (1.2)	0	13 (15.5)
Pneumothorax	84	3 (3.6)	2 (2.4)	1 (1.2)	0	6 (7.1)
Nausea	84	3 (3.6)	0	1 (1.2)	0	4 (4.8)
Hemoptysis	84	2 (2.4)	2 (2.4)	0	0	4 (4.8)
Chills	84	1 (1.2)	1 (1.2)	0	0	2 (2.4)
Anorexia	84	1 (1.2)	0	0	0	1 (1.2)

*Toxicity defined by National Cancer Institute Common Toxicity Criteria (grades 1–4).

†Total number of courses of Ad-p53 administered during the trial.

‡Highest grade toxicity associated with Ad-p53 treatment. Percentage of courses with this level of toxicity is shown in parentheses.

§Total number of each adverse event (grades 1–4) associated with Ad-p53. Percentage of courses with the adverse event is shown in parentheses.

transient hemoptysis were noted after bronchoscopic injection and were resolved with observation. The most common vector-associated adverse event was fever, occurring 6–24 hours after injection in 23 treatments (27.4%). These fevers were treated with antipyretics or observation and resolved within the next 24–48 hours. There was no increase in adverse events with repeat treatments or higher doses of Ad-p53, and dose-limiting Ad-p53 toxicity was not reached in this trial. In addition, no patient demonstrated hypotension or anaphylaxis despite repeated (up to six) doses of Ad-p53.

Effect on Tumor Growth

Clinical response of the injected tumor was evaluable in 25 patients (89%) and included the following: partial response in two patients (8%; 95% CI = 1%–26%), stabilization of disease in 16 patients (64%; 95% CI = 43%–82%) (range, 2–14 months), and progression of disease in seven patients (28%; 95% CI = 12%–49%). Three patients were not evaluable because they died from treatment problems unrelated to Ad-p53 before a 30-day follow-up CT scan was done. Of note, three of five patients who received less than 10^8 PFU of Ad-p53 showed continued progression of their disease while on treatment, whereas only four of 22 patients receiving 10^8 PFU or more showed disease progression. There was no clear relationship between patient characteristics, adverse events, gene expression, or tumor location/size/histology and clinical response. Details of the two patients (patients K and O) who demonstrated a partial response following Ad-p53 gene therapy are as follows:

Patient K presented with a left upper lobe large-cell carcinoma on November 1994. Because of poor results on pulmonary function tests, the patient was judged not to be a surgical candidate and was treated with 66 Gy of external beam radiation therapy. The primary tumor recurred in February 1996 and was treated with six cycles of paclitaxel and carboplatin. The patient was subsequently enrolled in the gene therapy protocol because the tumor had progressed. At a dose level of 10^9 PFU of Ad-p53, th tumor responded with a greater than 50% decrease in size (Fig. 2). No viable tumor was demonstrated on tumor biopsies after the first two courses of Ad-p53 therapy. After completion of gene therapy in June 1997, th patient was observed without further treatment and, at the time of the last follow-up (March 1998), showed no evidence of recurrent disease.

In September 1994, patient O was found by bronchoscopy to have an adenocarcinoma that was partially obstructing the left upper lobe. The patient was treated with two courses of cisplatin and etoposide, followed by 60 Gy of definitive external beam radiation therapy. In December 1995, the tumor recurred with bronchial obstruction of the left upper lobe and was treated with laser therapy and 21 cycles of mitomycin C and navelbin . One year later, in December 1996, the patient's left upper lobe of the bronchus was found to be reoccluded; laser therapy was attempted but failed. Direct intratumoral injection of 3×10^9 PFU of Ad-p53 was begun in December 1996, resulting in a partial response and reopening of the airway (Fig. 3). This response was maintained for 6 months with Ad-p53 alone. At the completion of therapy, residual tumor still remained and three additional courses of carboplatin and docetaxol were given, resulting in a complete histologic response. One year later, in December 1997, the tumor recurred, and the patient was begun on a follow-up Ad-p53 protocol, which was discontinued because of further tumor progression.

The median survival of all patients from the time of initiating gene therapy was 141 days (Fig. 4). At the time of last follow-up, five patients were still alive greater than 10 months after initiating therapy and two patients were being observed off all treatment without evidence of tumor growth.

Discussion

The estimated incidence of lung cancer in the United States in 1997 was 178 100, with more than 160 400 deaths *(29)*. Despite advances in chemotherapy, radiation therapy, and surgery, overall survival for this disease is still less than 13% *(29)*. Because of the poor results obtained with conventional therapy alone, additional treatment strategies are needed. Our study evaluated the novel strategy of intratumoral injection of an adenovirus vector expressing wt-p53 (Ad-p53) in patients with advanced NSCLC whose cancers had failed to respond to conventional treatments.

One important finding of this study was that multiple doses of Ad-p53 could be administered safely. We have reported previously *(28)*, in an article that described findings for 12 of the patients in this study, that neutralizing anti-adenovirus antibodies rise sharply in the serum after the first course of Ad-p53 and remain elevated throughout therapy. Despite this fact, we observed no major vector-related toxicity with repeat injections. A total of 84 doses of Ad-p53 were delivered; 56 of these doses were repeat injections (up to six injections given monthly). There were no anaphylactic reactions or episodes of hypotension and only one grade 3 vector-related adverse event (nausea) during treatment. All other toxic effects were grade 1 or 2 and consisted primarily of transient fevers treated with antipyretics. Repeated delivery of the vector by CT guidance or bronchoscopy was also feasible, and only six pneumothoraces developed during 84 injections. These pneumothoraces were treated with observation in four patients and with placement of a pigtail catheter in two patients. In addition, pain at the site of injection was noted in only 13 of 84 courses of therapy. Such pain was treated with analgesics and resolved within the first 24–48 hours of injection in most cases. Clayman t al. *(30)* also noted pain at the injection site as being the most common adverse event after intratumoral injection of Ad-p53 in patients with recurrent head and neck squamous cell carcinoma. In our study, a maximum tolerated dose was not reached. Dose escalation was limited by

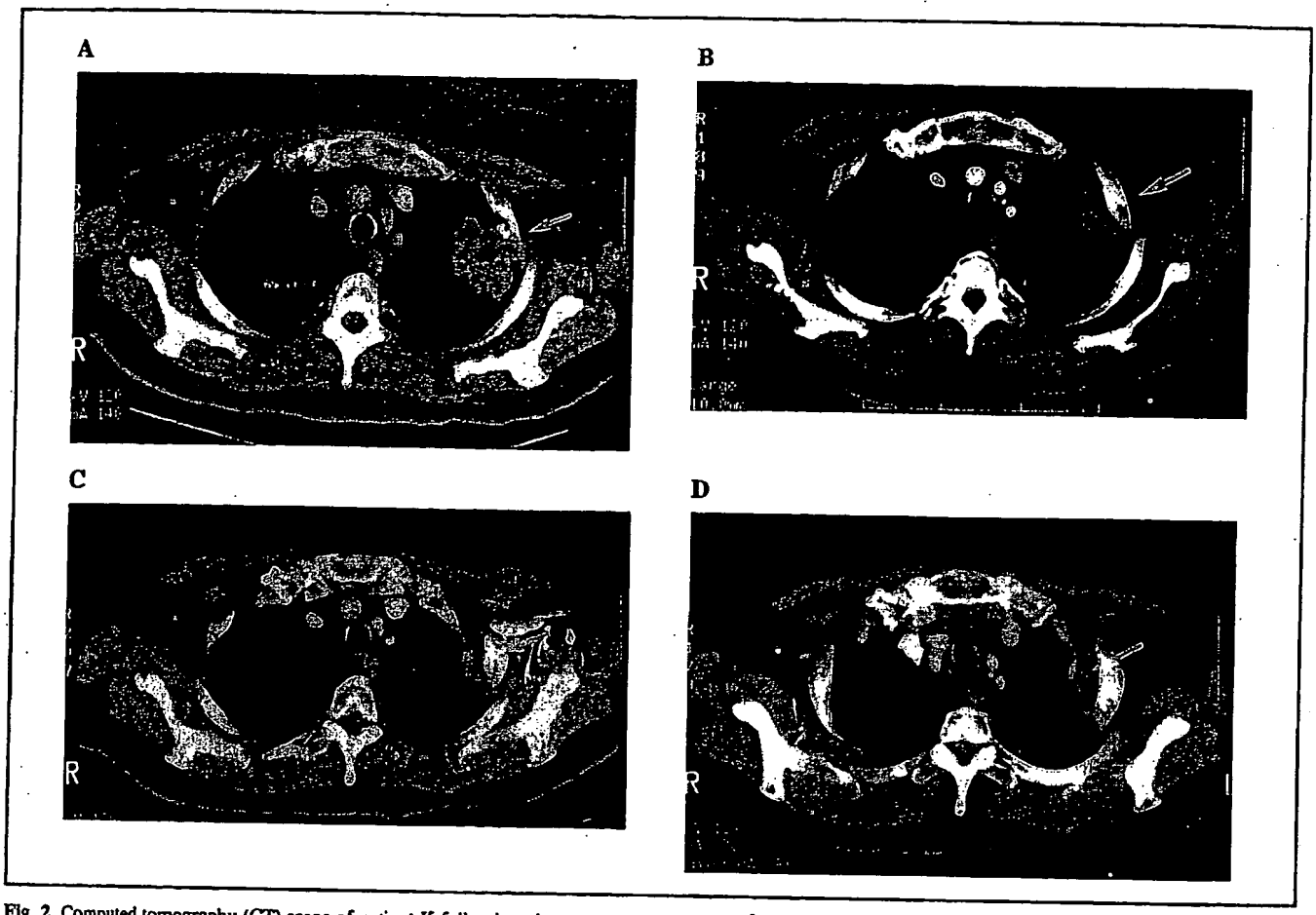

Fig. 2. Computed tomography (CT) scans of patient K following six courses of 10^9 plaque-forming units of Ad-p53, an adenovirus vector carrying the wild-type p53 complementary DNA. A) Before treatment, Arrow shows recurrent left upper lobe adenocarcinoma, which progressed after 66 Gy of external beam radiation therapy and six courses of paclitaxel and carboplatin (CT scan volume: $3 \times 4 \times 5$ cm = 60 cm^3). B) At 1 month after treatment, arrow shows tumor regression after one course of Ad-p53 treatment (CT scan volume: $2 \times 3 \times 5$ cm = 30 cm^3). C) At 8 months after treatment, image shows tumor regression following six courses of Ad-p53 gene therapy (CT scan volume: $2 \times 2 \times 3$ cm = 12 cm^3). D) Stable tumor 18 months after beginning treatment with Ad-p53 (CT scan volume: $2 \times 2 \times 3$ cm = 12 cm^3). No viable tumor was demonstrated during the last 4 months of therapy (14 sequential percutaneous biopsies), and the patient was observed off all treatment for 12 months without evidence of tumor progression.

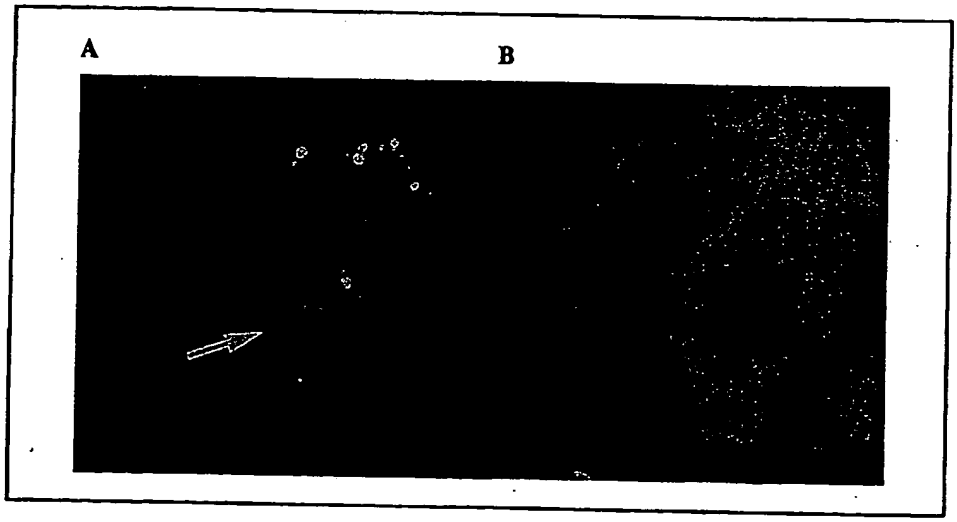

Fig. 3. Bronchoscopic images of patient O before treatment (A) and 30 days after treatment with 3×10^9 plaque-forming units of Ad-p53, an adenovirus vector that carried the complementary DNA encoding wild-type p53 protein (B). A) The tumor is obstructing the left upper lobe. B) Patient's left upper lobe airway 30 days after treatment. The response was maintained for 6 months with Ad-p53 alone.

the protocol to 10^{11} PFU. Similarly, Sterman et al. *(31)* found that a dose-limiting toxicity was not reached after intrapleural instillation of up to 10^{12} PFU of an adenovirus vector, Ad-HSV-tk, containing the herpes simplex virus–thymidine kinase gene into patients with mesothelioma. Because of the low toxicity, 10 patients at the nd of the study were able to be treated as outpatients rather than as inpatients, even though six of them were receiving the highest dose of Ad-p53. This low toxicity is im-

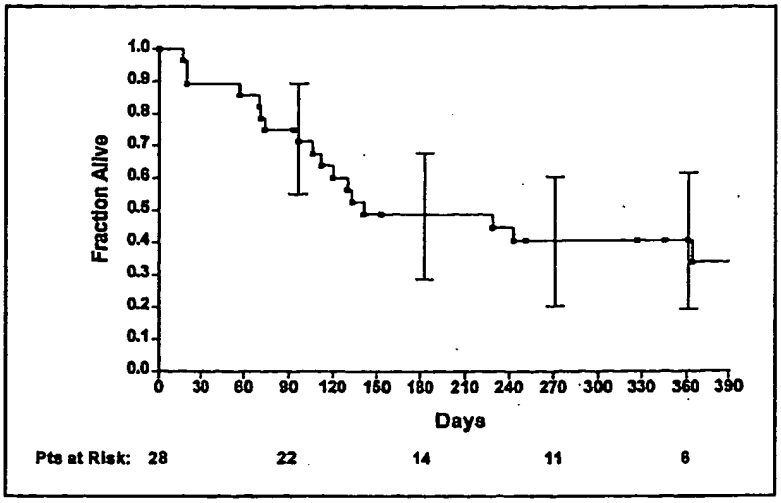

Fig. 4. Actuarial survival of all patients treated with Ad-p53, an adenovirus vector carrying the complementary DNA sequence encoding wild-type p53. The Kaplan–Meier method was used to assess survival (95% confidence intervals = error bars). Pts = patients.

portant for future trials because it means that Ad-p53 has potentially a high therapeutic index that allows it to be used in combination with other conventional treatments, such as chemotherapy, radiation therapy, or surgery.

The second important observation of this trial was that wt-p53 cDNA transfer and expression could be accomplished and documented in a large number of patients. We used vector-specific primers that incorporated flanking regions of the adenovirus to ensure detection of adenovirally transferred p53 mRNA rather than of native p53. Adenovirus-mediated p53 cDNA transfer appeared to be dose related. Transgene p53 expression could be documented by RT–PCR in 56% of the patients treated with 10^9 PFU or more, whereas only 30% of patients who received lower doses showed transgene p53 expression (Table 2). Importantly, Ad-p53 transgene expression could still be observed after multiple courses even in the presence of high serum levels of anti-adenovirus antibodies. Sterman et al. *(31)* also noted dose-related gene expression when mesothelioma patients were treated with intrapleural instillation of Ad-HSV–tk. In addition, Tursz et al. *(32)* documented increasing β-galactosidase expression in patients with endobronchial lung cancer following intratumoral injection of higher doses of an adenovirus vector containing the β-galactosidase gene. Since maximal adenovirus expression occurs *in vitro* at 3 days and drops off rapidly during the next week, our observation that transgene expression still occurs with multiple courses may be important for future clinical trials that require prolonged periods of transgene expression. Detection of gene expression following transfer of wt-p53 *in vivo* is difficult because successful transfer and expression of wt-p53 in a tumor may compromise evidence of gene expression by the rapid induction of apoptosis. Other constraints on detection of p53 transgene expression in this study are the small size and scant cellularity of the biopsy specimens and the low sensitivity of the RT–PCR assay *(33)*. These findings may explain in part the variation noted in PCR and RT–PCR assays performed on sequential biopsy samples.

The third important finding was that evidence of antitumor activity was suggested following Ad-p53 cDNA transfer. Two patients demonstrated a greater than 50% reduction in tumor size after Ad-p53 injection. In one patient, no viable tumor cells could be demonstrated in all biopsy specimens obtained during the last 4 months of treatment. Because of a lack of histologic evidence of cancer following treatment with Ad-p53 alone, we elected to observe this patient off all therapy; at last follow-up—more than 18 months after starting treatment—the patient was without evidence of recurrent tumor. Patients with endobronchial tumors may also represent a subset of patients who could benefit from Ad-p53 treatment, since we observed almost complete regression of a left upper lobe endobronchial tumor that had failed to respond to chemotherapy, radiation therapy, and laser treatment. To our knowledge, this is the first study to demonstrate sustained antitumor activity in NSCLC with gene transfer of wt-p53 alone. Since these patients had already failed to respond to multiple other treatments, future trials with untreated patients or with patients with earlier stage disease may have higher response rates. In addition, Clayman et al. *(30)* noted that Ad-p53 resulted in the partial regression of disease in two of 17 evaluable patients with recurrent head and neck carcinoma. These results suggest that the antitumor activity noted with Ad-p53 treatment may be effective in other types of cancer. Because of heterogeneity in patients and tumors entered in this phase I study, definitive statements about clinical efficacy are difficult. No clear association could be demonstrated between patient and tumor characteristics and response; however, it did appear that higher doses of Ad-p53 ($>10^8$ PFU) were associated with longer times to disease progression. It is interesting that both patients who responded demonstrated continued disease regression with repeated administrations of Ad-p53, even in the face of elevated anti-adenovirus antibodies. Li et al. *(34)* have shown in an immunocompetent mouse model that multiple intratumoral injections of Ad-p53 result in increased tumor regression and transgene expression despite elevated levels of circulating adenovirus antibodies. These observations validate the strategy of administering multiple intratumoral injections of Ad-p53 to maximize transgene expression and tumor response.

Although immune-mediated responses have been reported after adenovirus treatment *(35)*, we observed no evidence of a substantial increase in inflammatory cell infiltrates in posttreatment tumor biopsy specimens. In addition, despite increases in neutralizing levels of anti-adenovirus antibodies and increased lymphocyte proliferative responses to adenovirus serotype 5 antigens, no antibody-dependent cytotoxicity could be demonstrated in posttreatment serum samples, and changes in lymphocyte proliferative responses to p53 mutant and wild type were not observed (Yen N, Ioannides CG, Xu K, Swisher SG, Lawrence DD, Kemp BL, et al.: unpublished observations).

Moreover, preclinical studies have demonstrated in both immunocompetent and immunodeficient animal models an antitumor activity that appears to be p53 specific *(7,8,10,12)*. It is unlikely, therefore, that the antitumor activity we observed in this trial was due to immune-mediated effects. Another possible mechanism for the antitumor effects is the induction of apoptosis by Ad-p53. We have observed that the transduction of human lung cancer cells with Ad-p53 results in the increase of the pro-apoptotic Bcl-2 family members Bax and Bak *(36)*. This mechanism is supported by the high levels of apoptosis seen with TUNEL staining in the posttreatment tumor biopsy samples from 11 of 24 evaluable patients. In the two patients who responded to Ad-p53, one patient demonstrated a posttreatment AI of 46%, while the other patient could not be evaluated because of the large amount of necrosis after treatment. In addition, since posttreatment tumor biopsies were always performed 3 days after Ad-p53 injection, the critical period of apoptosis may have been missed in some patients because *in vitro* studies *(37)* suggest that apoptosis can occur as soon as 3 hours after induction. It is interesting, however, that, in those patients who demonstrated TUNEL activity, TdT expression was markedly higher than that reported in patients following chemotherapy. Ueda et al. *(38)* noted only two of 22 patients with an AI greater than 10% after intra-arterial infusion of chemotherapy in cervical carcinoma, whereas we noted four patients with an AI ranging from 47% to 87% after Ad-p53 treatment (Table 2). Intratumoral injection of retroviral p53 has also been associated with increased TUNEL activity *(14)*, which suggests that induction of apoptosis by transgene expression of wt-p53 may be one of the mechanisms underlying tumor regression.

In summary, this study demonstrates for the first time, to our knowledge, the clinical feasibility of adenoviral p53 cDNA transfer strategies in NSCLC. The safety and efficacy of repeated doses, even in large, established tumors, suggest that a therapeutic window exists during which clinical benefit is not accompanied by additional toxicity. Several potential clinical applications of this technology exist. At the present time, local control of lung cancer remains suboptimal. Radiation therapy is limited in effectiveness because of toxicity to normal tissues at higher doses. Since preclinical studies suggest synergism between radiation and Ad-p53 without increased toxicity, one potential strategy might be to combine the two modalities for enhanced local tumor control *(39)*. Another possibility is in premalignant lesions, such as bronchial dysplasias, where p53 mutations are known to precede invasive carcinoma *(40,41)*. Localized injection of a nontoxic agent such as Ad-p53 might ultimately play a role in preventing the development of invasive cancer. Phase II clinical trials are now under way to determine the feasibility of these strategies and to determine the clinical role of Ad-p53 in the treatment of lung cancer.

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NOTES

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